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Although most women with breast cancer present with local disease, 30-40% of these women will develop advanced disease. Several tumor characteristics have been suggested to be predictive of disease recurrence, among them the presence of micrometastatic deposits of breast cancer cells in the patient's bone marrow. The purpose of this research project is to determine if adjuvant hormonal therapy or chemotherapy can alter this risk factor, that is, will standard adjuvant therapy eliminate these metastatic tumor cells. Additionally, this project is seeking to determine if a patient in whom these cells are not eliminated has a greater risk of tumor recurrence than the patient in whom the micrometastatic cells are eliminated. To accomplish this project bone marrow aspirates are being screened for micrometastases using highly sensitive immunological techniques prior to the initiation of and after completion of adjuvant therapy. The patient's course (disease free and overall survival) will then be followed for at least five years. Six patients are currently enrolled but it is too early to have any post-therapy results. This project is also exploring the effect of therapy on marrow micrometastases in patients with advanced disease in a similar manner.

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INTRODUCTION

Breast cancer. The American Cancer Society estimates there will be 183,000 women who will hear that diagnosis in 1993. Breast cancer is the second most common malignancy in women and it killed 40,000 women in 1991 (1). The risk for breast cancer continues to increase, such that one in eight women in the United States will develop breast cancer during their lifetime. Currently, approximately 40% of women with breast cancer present with disease confined to the chest and another 40% of women present with disease confined to the chest and axillary lymph nodes. These women are treated with surgery, radiation therapy and subsequent adjuvant therapy. Despite these therapeutic interventions, metastatic disease will develop in approximately 30-40% of both groups of these women (2).

Several characteristics of breast cancer have been suggested to be risk factors for breast cancer recurrence and decreased survival. Among these characteristics are the size, histology, and hormonal status of the original tumor and the presence of positive lymph nodes or metastases (3). Although these factors have helped determine the breast cancer populations at risk for recurrence or early death, they are not sufficient to define the risks for an individual patient. Evidence is accumulating that other features of a patient's tumor, such as S-phase analysis (4), expression of selected oncogenes (5), and the presence of bone marrow micrometastases (BMM), may have prognostic significance for that patient's individual risk of recurrence and survival (6-8).

Redding et al from the Ludwig Institute for Cancer Research (United Kingdom) examined bone marrow from 110 patients with primary breast cancer for the presence of micrometastases (6). Bone marrow was obtained from each patient (2-4 ml aspirate) from several sites. Mononuclear and malignant cells were isolated by Ficoll gradient centrifugation, placed onto glass slides and wet-fixed in absolute ethanol. The slides were then stained immunocytochemically with an antibody probe which recognized an epithelial membrane antigen present on breast cells in order to detect the presence of breast cancer cells. Breast carcinoma cells were detected in the bone marrows from 31 of the 110 patients (28%). None of these patients had malignant cells in their marrow as detected by conventional Giemsa staining.

In a follow-up report, 81 of 307 patients (26.4%) evaluated as outlined above had bone marrows positive for micrometastases (7). The correlations previously noticed between the presence of BMM, advanced T stage, and vascular invasion became stronger, reaching P values <0.05. Additionally, it was noted that although only 19% of lymph node negative patients had BMM, 33% of lymph node positive patients had BMM ($p=0.013$). With a median follow-up time of 27 months, 32 of the 81 patients with positive marrows (29.5%) had recurrent breast cancer, while only 45 of 226 (19.0%) whose marrows were antibody negative had recurred. A total of 36 patients died. Of these, 15 deaths occurred in the group of 81 patients who had BMM at presentation (18.5%), and the other 21 deaths occurred in the 226 initially free of BMM (9.3%). The difference in the two groups reached a P value of < 0.05. However, BMM could not be shown to be an independent prognostic factor for either disease free survival (DFS) or overall survival (OS).

Cote et al from Memorial Sloan-Kettering recently reported follow-up data (median follow-up of 29 months) on their patients evaluated for BMM (8). Recurrences occurred in 7 of the 18 patients with BMM at initial presentation and in 5 of the 31 patients without BMM. The estimated 2-year recurrence rate in patients with BMM was

33% and for those patients without BMM, it was 3%. When BMM status was combined with lymph nodal (LN) status, the difference in recurrence rates became even more striking. Those patients who were BMM+ and LN+ had a 42 percent 2-year recurrence rate, while those who were BMM- and LN- had a zero 2-year recurrence rate. This data indicated that the combination of positive BMM status and positive nodal status was a significant prognostic indicator for early recurrence. No overall survival data was presented.

The above studies suggest several interesting events in breast cancer. First, the data from Cote et al (8) suggests that patients with BMM are at risk for early recurrence. Second, BMM occur very early in certain, but apparently not all, patients, as evidenced by finding BMM in Stage I breast cancer patients. Third, BMM probably occur as a result of hematogenous spread, as evidenced by finding BMM in lymph node negative patients.

Although the available data suggests that BMM may have an effect on DFS and OS, the effects of adjuvant therapy (chemotherapy or hormonal therapy) on the presence of BMM remains unclear (9). The status of BMM in relapsed patients and the effects of therapy on BMM in these patients has not been reported.

The purpose of this work is therefore twofold. One, to determine if hormonal therapy or chemotherapy will eradicate BMM in women with breast cancer in either the adjuvant or advanced disease setting. Two, to determine if failure to eradicate BMM with systemic therapy is a prognostic factor for decreased disease-free survival or overall survival.

The method of approach is to enroll eligible women with breast cancer into the study protocol where they have bone marrow aspirates done prior to the beginning of hormonal or chemotherapy and at specific times after their therapy in order to look for BMM using antibodies which recognize breast cancer cells. The pre- and post-therapy presence or absence of BMM is then correlated with the disease-free and overall survival in this group of breast cancer patients.

METHODS

Patient characteristics. Women who were: (1) between the ages of 18 and 70 years with newly diagnosed or recurrent breast cancer, (2) were to receive hormonal or chemotherapy and (3) gave informed consent, were eligible for the study. Patients were entered into the study within two months of primary diagnosis or recurrence. Patients with prior radiation to the iliac crests or who had known metastatic disease to the iliac crests were excluded. Bone marrow samples (2-3 mls per iliac crest) from eligible patients were obtained at the time of their entry onto the study. Bone marrow apirates will be repeated after completing each chemotherapy regimen, after six months of hormonal therapy, or after a change in hormonal therapy. All treatment, clinical follow-up, and other care decisions remained the responsibility of the patient and her physicians.

Prestudy evaluation. All patients had a physical examination, chest x-ray, bone scan, CBC, electrolytes, BUN and creatinine, and liver function test panel.

Marrow processing. The marrow samples were diluted with PBS and layered onto a Ficoll-Hypaque density gradient and centrifuged. The cells at the interface layer were collected and then washed three times with RPMI-1640 plus 5% FCS. The cells

(which consist of mononuclear cells and tumor cells) were then suspended at 1 x 10^7 cells/ml in PBS and placed, by single drops, onto microscope slides and dried. Two slides from each patient were stained with Wright's stain for cytological examination. Ten to twelve slides from each patient were used for identification of breast cancer cells by indirect immunoglucose oxidase studies (10,11). The anti-cytokeratin mouse monoclonal antibody 35ßH11 (provided by Dr. Allen Gown) was used as the primary antibody to detect the presence of breast cancer cells in the bone marrow (10,12).

Indirect immunoglucoseoxidase assay. Slides with cells were incubated with a primary antibody for 30-60 minutes, followed by washing 3 times with PBS. Specimens were then incubated 30 minutes with a glucose oxidase conjugated secondary antibody (Cappel, Durham, NC). Next, the specimen was washed 3 times with PBS and incubated with the chromogen phenazine methosulfate/two,2-di-p-nitrophenyl-5,5-diphenyl-3,3'(3,3-dimethoxy-4,4'-diphenylene)-ditetrazolium chloride (PMS/NBT) for 8-10 minutes. The glucose oxidase enzyme label oxidizes the glucose substrate resulting in the reduction of NBT through the intermediate electron carrier PMS. Upon reduction of NBT, a highly colored, insoluble product is formed which allows the localized glucose oxidase to be visualized. Slides were rinsed in distilled water, counterstained with methyl green for 5 minutes, dehydrated and coverslips applied. Control slides included specimens treated with (1) no primary antibody, (2) normal peripheral blood, and (3) normal peripheral blood seeded with MCF-7 cells (11).

Statistical Analysis. Tumor staging, histology, and hormonal status were obtained from pathology and surgical reports. Hospital and clinic records were reviewed to obtain data on patient's clinical course to include treatment, DFS and OS. The Chi-squared test will be used to evaluate the relationship between the presence of BMM and other known prognostic factors. Standard survival analyses will be used to evaluate the relationship between BMM and DFS and OS.

RESULTS

To date, six patients have been enrolled on the study. An example of the data collection form and a completed patient data form is provided in Appendix 1 and 2, respectively. Figure 1 shows a MCF cell mixed with normal peripheral blood cells and positively stained with the anticytokeratin antibody, 35ßH11.

DISCUSSION

The natural history of breast cancer is not well understood and consequently, many patients receive therapy that does not work for them (as evidenced by relapses) while other patients receive treatment they may not need (which patients really benefit from years of adjuvant tamoxifen?). By learning more about the biology of breast cancer and its outcome in the individual patient, the treatment of breast cancer can be refined and tailored for the individual patient. This will help to ensure that an optimal regimen is utilized and eliminate unnecessarily treating certain patients. BMM is thought to be a prognostic indicator of increased risk of breast cancer recurrence. The purpose of this research project is to determine if adjuvant hormonal therapy or chemotherapy can alter this risk factor, that is, will standard adjuvant therapy eliminate

these metastatic tumor cells. Additionally, this project is seeking to determine if patients in whom these cells are not eliminated have a greater risk of tumor recurrence than the patients in whom the micrometastatic cells are eliminated. To accomplish this project, bone marrow aspirates are being screened for micrometastases using highly sensitive immunological techniques prior to the initiation of and after completion of adjuvant therapy. The patient's course (disease free and overall survival) will then be followed for at least five years. This project is also exploring the effect of therapy on marrow micrometastases in patients with advanced disease in a similar manner. Six patients have been enrolled to date, but given that none of them have had evidence of BMM, no observation of the effect of therapy upon BMM has been possible.

CONCLUSIONS

We have recognized that our patient accrual has been less than anticipated. We have made two significant changes to our protocol. We have added the University of Washington as a second source of patients and we have amended the study protocol so that the bone marrow aspirates can be done in the operating room during the patients diagnostic/definitive surgical procedure. These changes, as well as approval from the Institutional Review Board at Madigan Army Medical Center, have already been forwarded to Medical Research and Development Command and should increase our enrollment during our second year of patient accrual.

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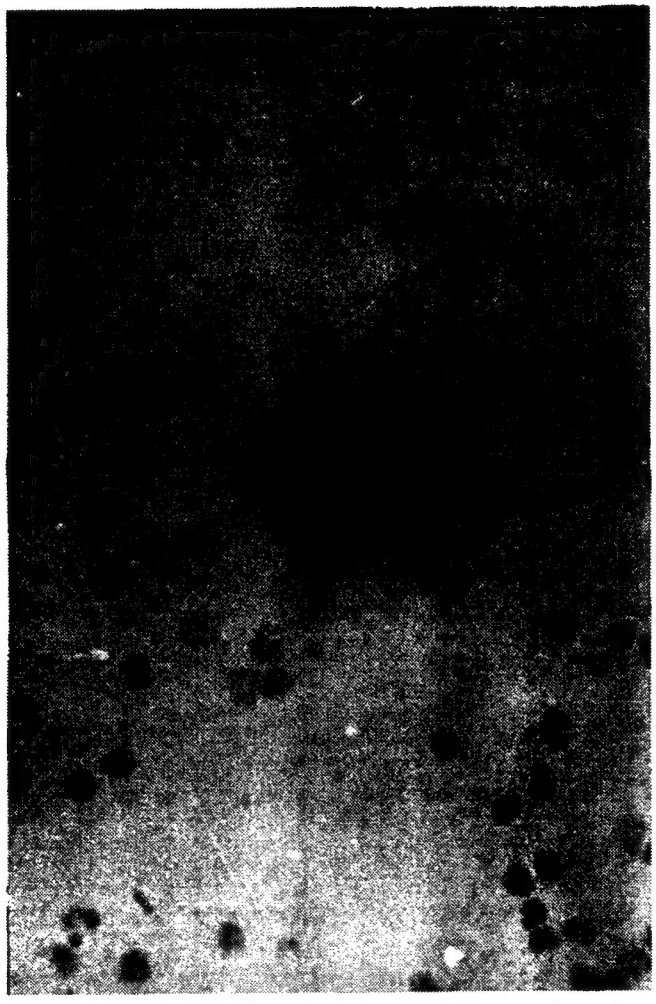

Figure 1. An MCF-7 cell mixed with normal peripheral blood cells and positively stained with the anticytokeratin antibody, 35β11.

APPENDIX 1

Example of the data collection form

BMM STUDY DATA '93-94 : LOCAL DISEASE

	PRE TREATMENT	TREATMENT	POST TREATMENT
1. Study Entry Number			
2. Date of entry			
BMM result			
4. # positive cells			
5. Histology results (BM)			
6. Name			
7. SSN			
8. DOB			
9. Race			
10. Gender			
11. Date of initial dx			
12. Primary site / laterality			
13. Histology & grade (tumor)			
14. Tumor size			
15. Date of bx			
16. Bx accession no.			
17. Primary surgical procedure/date			
18. Surgical accession no.			
19. Regional nodes pos./ exam			
20. AJCC staging system (staging basis; elements- TNM;stage group)			
21. ER value/interp			
22. PR value/interp			
23. CXR result			
24. MMG result			
25. Bone scan			
26. WBC			
27. HGB/HCT			
28. Platelets			
29. Electrolytes			
30. BUN/Ca			
31. AST			
32. ALT			
33. ALK PHOS			
34. Gamma GT			
35. T Bili			

36. Date/dose of rad. tx			
Date/type of first chemo/ horm tx			
38. Date/type/sites of 1st recurrence			
- tissue bx confirmation			
39. Date/ type of tx for recurrence or peristent dx			
40. Date of last contact or death			
41. Status of patient/cancer			

APPENDIX 2

Example of completed patient data collection form

BMM STUDY DATA '93-94 : LOCAL DISEASE

	PRE TREATMENT	TREATMENT	POST TREATMENT
1. Study Entry Number	4		
2. Date of entry	27Sep93		
3. BMM result	neg		
4. # positive cells	none		
5. Histology results (BM)	no tumor present		
6. Name	Uyechi, Hidena		
7. SSN	[redacted]		
8. DOB	[redacted]		
9. Race	Japanese		
10. Gender	female		
11. Date of initial dx	2Sep93		
12. Primary site / laterality	breast / left		
13. Histology & grade (tumor)	invasive lobular carcinoma		
14. Tumor size	1.2cm		
15. Date of bx	2Sep93		
16. Bx accession no.	S-9876-93		
17. Primary surgical procedure/date	L breast mastectomy / 27Sep93		
18. Surgical accession no.	S-10814-93		
19. Regional nodes pos./ exam	0 / 10		
20. AJCC staging system (staging basis; elements- TNM;stage group)	$T_1N_0M_0$		
21. ER value/interp	315fm/mg / pos		
22. PR value/interp	<1.0fm/mg / neg		
23. CXR result	normal		
24. MMG result	L-abn / R-norm		
25. Bone scan	anterior 7th rib-hot spot (trauma)		
26. WBC	9.9		
27. HGB/HCT	10.2 / 29.2		
28. Platelets	188 x 10 ^3		
29. Electrolytes	Na-134, K-3.8, Cl-94, CO_2-24		
30. BUN/Ca	7 / na		
31. AST	28		
32. ALT	32		
33. ALK PHOS	77		
34. Gamma GT	33		
. T Bill	1.3		

36. Date/dose of rad. tx	none		
Date/type of first chemo/ horm tx		8Oct93 / tamoxifen	
38. Date/type/sites of 1st recurrence	none		
- tissue bx confirmation			
39. Date/ type of tx for recurrence or peristent dx	n/a		
40. Date of last contact or death		Oct93	
41. Status of patient/cancer		alive	